Serpent regulates *Drosophila* immunity genes in the larval fat body through an essential GATA motif

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Insects possess a powerful immune system, which in response to infection leads to a vast production of different antimicrobial peptides. The regulatory regions of many immunity genes contain a GATA motif in proximity to a κ B motif. Upon infection, Rel proteins enter the nucleus and activate transcription of the immunity genes. High levels of Rel protein-mediated *Cecropin A1* expression previously have been shown to require the GATA site along with the κ B site. We provide evidence demonstrating that the GATA motif is needed for expression of the *Cecropin A1* gene in larval fat body, but is dispensable in adult fat body. A nuclear DNA-binding activity interacts with the *Cecropin A1* GATA motif with the same properties as the *Drosophila* GATA factor Serpent. The GATA-binding activity is recognized by Serpent-specific antibodies, demonstrating their identity. We show that Serpent is nuclear in larval fat body cells and haemocytes both before and after infection. After overexpression, Serpent increases *Cecropin A1* transcription in a GATA-dependent manner. We propose that Serpent plays a key role in tissue-specific expression of immunity genes, by priming them for inducible activation by Rel proteins in response to infection.

Keywords: cecropin/fat body/GATA motif/innate immunity/Serpent

Introduction

The invertebrate immune system is non-clonal and in many ways analogous to the innate immune system of vertebrates. The relationship between the immune systems of such diverse phylogenetic groups as insects and mammals has become increasingly evident during the last few years (for a recent review see Medzhitov and Janeway, 1997). In insects, the immune response is induced rapidly upon injury or infection by bacteria, fungi and other pathogens (reviewed in Engström, 1999). Infections of the

animal lead to a vast production of antimicrobial peptides such as attacins, cecropins, defensins and dipterin (reviewed in Hultmark, 1993; Boman, 1995; Hoffmann and Reichart, 1997). The genes coding for these peptides are regulated at the level of transcriptional initiation. Several conserved *cis*-elements have been found in the upstream region of genes that are known to play a role in host defence. The most prominent one is the κ B-like element, which has been found in the regulatory regions of all cloned inducible insect immune genes (reviewed in Engström, 1998). In mammals, κ B motifs are known to bind transcription factors belonging to the Rel family, such as NF- κ B (Leonardo and Baltimore, 1989), and to influence genes of the immune system (Stancovski and Baltimore, 1997). In *Drosophila*, the κ B-like sequence is bound by the Rel proteins dorsal, dorsal-related immunity factor (Dif) and Relish (Steward, 1987; Ip *et al.*, 1993; Dushay *et al.*, 1996).

The phenotypes of different mutants affecting the immune response in *Drosophila* suggest that at least two different signalling pathways are utilized for the induction of antimicrobial peptide genes (Ip *et al.*, 1993; Lemaitre *et al.*, 1995, 1996; Corbo and Levine, 1996; Williams *et al.*, 1997; Wu and Anderson, 1998). It was proposed that one pathway is responsible for the induction of the antibacterial peptides, the other for the induction of the antifungal peptides (Lemaitre *et al.*, 1996). In addition, it was found recently that the antimicrobial response can discriminate between various classes of microorganisms (Lemaitre *et al.*, 1997).

A large number of insect immunity genes contain a GATA motif (WGATAR) situated close to the κ B motif in their regulatory regions (Kadalayil *et al.*, 1997). Both the κ B and the GATA motifs have been shown to be necessary for full *Drosophila Cecropin A1* (*CecA1*) promoter activity in transfection assays (Engström *et al.*, 1993; Petersen *et al.*, 1995; Kadalayil *et al.*, 1997; Roos *et al.*, 1998). By using P-element transformation, we demonstrate in this study that the GATA motif is crucial for the tissue-specific expression of the *CecA1* gene in the larval but not in the adult fat body.

The cell line malignant blood neoplasm-2 (mbn-2) is of haemocyte origin (Gateff *et al.*, 1980). Treatment of these cells with lipopolysaccharide (LPS) induces an immune response (Samakovlis *et al.*, 1992). In the nuclear fraction of mbn-2 cells, we previously identified a DNA-binding activity that specifically interacts with the *Drosophila* GATA consensus sequence (Kadalayil *et al.*, 1997). We postulated this GATA-binding activity (GBA) to be a member of the GATA family of transcription factors. GATA transcription factors have been found in many different organisms spanning from yeast to man. In mammals, the GATA motif is found in promoters of erythroid-expressed genes, and the GATA transcription factors are

essential for haematopoiesis (Orkin, 1995). In *Drosophila*, three members of this family have been reported: *pannier* (*pnr* or dGATAa) regulates the *achaete* and *scute* complex (Romain *et al.*, 1993; Winick *et al.*, 1993), *serpent* (*srp* or dGATAb) is necessary for development of many organs, among those the larval fat body and embryonic blood cells (Abel *et al.*, 1993; Rehorn *et al.*, 1996; Sam *et al.*, 1996), and dGATAc, which also is implicated in organogenesis in early embryos (Lin *et al.*, 1995). A partial *srp* cDNA, named *abf*, was cloned originally in a screen for factors binding to regulatory regions of the fat body-expressed *Drosophila* alcohol dehydrogenase (*Adh*) gene. This cDNA encoded a truncated Srp protein called the A-box-binding factor (ABF). In co-transfection assays, ABF was able to activate an artificial *Adh* promoter (Abel *et al.*, 1993). Recently, Rehorn *et al.* (1996) cloned the *Drosophila* *srp* locus and isolated a full-length cDNA.

On the basis of the biochemical properties of the GBA, we decided to test Srp experimentally as a potential *trans*-activator of the *CecA1* gene. In this study, we show for the first time that the Srp protein is present in tissues with known immunocompetence, such as the larval fat body, where we also show that the GATA motif is essential for *CecA1* expression. Larval fat body extracts contain GATA-binding activities. We demonstrate that Srp is identical to or at least a component of the GBA in both mbn-2 cell and larval fat body nuclear extracts. Finally, Srp acts as a positive regulator of the *CecA1* gene and is therefore likely to play a key role in the regulation of immunity genes.

Results

The GATA motif is essential for expression of the *CecA1* gene in the larval fat body

The importance of the κ B-motif for expression of *Drosophila* immunity genes has been studied both in transgenic animals and in transfection assays (Engström *et al.*, 1993; Kappler *et al.*, 1993; Meister *et al.*, 1994; Petersen *et al.*, 1995; Gross *et al.*, 1996; Roos *et al.*, 1998). The GATA motif has been demonstrated to be necessary for expression of *CecA1* reporter constructs in mbn-2 cells (Kadalayil *et al.*, 1997). To investigate the function of the GATA motif for tissue-specific expression *in vivo*, we generated transgenic flies carrying the pA16 *CecA1-lacZ* reporter construct, in which the GATA core sequence was altered to CGAG (Figure 1). Transgenic animals carrying the pA16 or the unmodified pA10 reporter construct (Figure 1) were challenged with LPS and the reporter expression was analysed using X-gal as a chromophore. Larvae carrying the pA10 reporter construct mounted strong induction of the reporter gene in the fat body upon LPS injection (Engström *et al.*, 1993; Roos *et al.*, 1998; Figure 2C). Staining of tissues from transgenic larvae carrying the pA16 construct was negative in both unchallenged (Figure 2A) and LPS-treated animals (Figure 2B). In LPS-injected adults, on the other hand, the pA16 construct conferred normal levels of β -gal expression in the fat body (Figure 2E and G). In uninjected adults, only tissues known to possess endogenous β -gal activity stained blue with X-gal (Figure 2D and F). We conclude that the GATA motif is necessary for expression of the *CecA1*

Fig. 1. Schematic representation of the *CecA1-lacZ* fusion constructs pA10 and pA16 and the expression plasmid pAct-*srp*. The pA10 and pA16 constructs contain upstream regions of the *CecA1* gene and the transcriptional start site (arrow) fused to an SV40 leader, providing a translational start site in-frame with the *E. coli lacZ* coding sequence (grey box). Numbers refer to position relative to the CAP site. Plasmid pA16 carries mutations in the GATA core sequence (GATA to CGAG). The expression plasmid pAct-*srp* carries the Srp cDNA under the control of the constitutive *Drosophila* actin 5C promoter region.

gene in the larval fat body but not needed for expression in the adult fat body.

Srp binds to the *CecA1* GATA motif

We previously demonstrated the presence of a specific GBA in the nucleus of mbn-2 cells (Kadalayil *et al.*, 1997; Figure 3A, lane 1). Our results suggested that the GBA belongs to the family of GATA transcription factors. The embryonic expression pattern of *srp* and the fact that *srp* mutants fail to develop fat body and haemocytes led us to investigate Srp as a potential regulator of *Drosophila* antimicrobial genes. To analyse the DNA-binding properties of Srp, electrophoretic mobility shift assays (EMSAs) were performed with *in vitro* transcribed and translated *srp* cDNA. A protein-DNA complex was formed when *in vitro*-translated Srp was incubated with a 32 P-labelled oligonucleotide containing the *Drosophila* *CecA1* GATA site (Figure 3A, lane 2). The Srp-containing complex co-migrated with the GBA (Figure 3A, compare lanes 1 and 2), suggesting that the protein composition is similar or identical in the two complexes. The presence of Srp protein in the *in vitro* translated sample was confirmed by Western blot analysis developed with a Srp-specific antibody (Figure 4, lane 2). Mock-translated wheat germ extract was also examined for DNA-binding activity and was found to be negative (Figure 3A, lane 6).

The Srp DNA-binding activity was competed effectively by the unlabelled GATA oligonucleotide (Figure 3A, lane 3), but not competed by an oligonucleotide (m1) in which the GATA core sequence had been changed from GATA to CGAG (Figure 3A, lane 4). The conserved GATA motif found in the 5' region of the *Drosophila* *Cec* genes and the dipterin gene is extended on the 3' side by three bases (A_4 GATAA T / $_G$ G C / $_T$) (Kadalayil *et al.*, 1997). Competition experiments using an unlabelled oligonucleotide in which GC on the 3' side has been mutated to TG, keeping the

Fig. 2. The GATA motif is necessary for expression of the *CecA1* gene in the third larval stage but not in the adult stage. (A–G) X-gal staining of tissues from transgenic larvae carrying the pA16 (A and B) or the pA10 (C) constructs and whole transgenic adults carrying the pA16 construct (D–G). (F) is an enlargement of (D), and (G) is an enlargement of (E). (B), (C) and (E) were injected with LPS (10 µg/ml) into the body cavity 3–6 h before staining. All tissues were stained overnight. fb, fat body; sgl, salivary gland; mg, midgut. Six independent transgenic strains of pA16 were generated. Five of these strains were homozygous viable and the induction pattern in the larval and adult fat body was analysed in these five strains. None of the strains showed any β -gal activity in the larval fat body, with or without pre-treatment with LPS. Four strains displayed strong induction of the reporter gene in the adult fat body after injection with LPS. One strain did not show any expression in the adult fat body even after immune challenge. This variability presumably is caused by the influence of nearby sequences or chromatin structure (Spradling and Rubin, 1982). One strain was homozygous lethal and was not analysed further.

core sequence intact (m2), competed as efficiently as the wild-type (Figure 3A, lane 5). The DNA-binding properties of Srp are the same as those previously determined for the GBA, supporting our theory that Srp protein is a constituent of the GBA (Kadalayil *et al.*, 1997).

Srp is a component of the GBA

To investigate further the presence of Srp in the GBA, nuclear extracts were pre-treated with two different antisera, S1 [directed against the central domain of Srp including the DNA-binding Zn finger (Sam *et al.*, 1996)] and S2 [directed against the C-terminal 22 amino acids of Srp (Hu, 1995)]. The extracts were then incubated with a 32 P-labelled oligonucleotide containing the *Drosophila*

CecA1 GATA motif in order for DNA binding to take place. In the nuclear fraction of untreated and LPS-treated mbn-2 cells, a strong band shift was present, GBA (Figure 3B, lanes 1 and 2). Pre-treatment of the nuclear extracts with the S1 antiserum disrupted the GBA (Figure 3B, lane 3), while the S2 antiserum resulted in a supershift (Figure 3B, lane 4). The two antisera, S1 and S2, were also incubated with oligonucleotide alone to rule out any direct interaction between the antibodies and the oligonucleotide (Figure 3B, lanes 5 and 6). Pre-treatment of the extract with normal serum did not influence the migration of the GBA (Figure 3B, lane 7). We conclude that Srp is, if not identical to, at least a component of the GBA.

Fig. 3. Srp binds specifically to the *CecA1* GATA motif and is a component of the GBA. (A) Electrophoretic mobility shift assay (EMSA) with *in vitro* translated *srp*. DNA binding was carried out with nuclear extract (N) (lane 1) or *in vitro* translated Srp (lanes 2–5) and a 32 P-labelled GATA probe (wt). Unlabelled competitors were added to the reactions in lanes 3–5 as indicated (for details, see Materials and methods). As a negative control, the wheat germ extract (WG) was analysed for the presence of GATA-binding activity (lane 6). (B) EMSA with nuclear extract (N) from untreated (lane 1) or LPS-treated (lane 2) mbn-2 cells incubated with a 32 P-labelled GATA probe (wt). LPS-treated nuclear extract was pre-incubated with antisera against the DNA-binding domain of Srp (S1) (lane 3), with antisera against the C-terminus of Srp (S2) (lane 4) or with normal rabbit serum (NS) (lane 5). The Srp antibodies S1 and S2 were also incubated with the probe alone (lanes 5–6). The asterisk indicates the supershift of the GBA. The protein–DNA band indicated by an arrowhead is due to unspecific binding.

Expression of *srp* in an immunoresponsive *Drosophila* blood cell line

Previous studies have shown that *srp* mRNA is expressed throughout development (Abel *et al.*, 1993; Winick *et al.*, 1993). The *srp* gene is expressed from the blastodermal stage and is essential for the differentiation of haemocytes (Rehorn *et al.*, 1996). Studies on expression of *srp* in haemocytes at later stages have not been reported. The mbn-2 cell line is of haemocytic origin (Gateff *et al.*, 1980) and expresses the antimicrobial peptide genes when treated with LPS. Two antisera with different specificities against Srp reacted with the GBA in this cell line (Figure 3B). To investigate the biochemical properties of Srp in the mbn-2 cell line, we performed Western blot

Fig. 4. Srp is present in different forms in mbn-2 cells. Western blot analysis with the S2 Srp antiserum. Total cell extracts (T) prepared from mbn-2 cells were analysed for Srp content (lane 1). Several protein bands were identified by the S2 antiserum. The lower band denoted Srp corresponds to a 130 kDa protein and co-migrates with *in vitro* translated Srp (lane 2). The Srp-specific bands indicated by an arrowhead are due to phosphorylation of the 130 kDa form. The upper band denoted Srp' indicates the 170 kDa form of Srp. In the mock-translated wheat germ extract (WG), no protein was recognized by the antiserum (lane 3). A parallel Western blot was analysed with another Srp antibody (S1), revealing an identical pattern of bands and thus verifying their identity as Srp (data not shown).

analysis on total cell extracts (Figure 4, lane 1) and on *in vitro* translated Srp (Figure 4, lane 2). The predicted molecular weight of Srp is 102 kDa. Several protein bands were observed in mbn-2 cell extracts using the S2 antiserum (Figure 4, lane 1); incubation with the secondary antibody alone did not generate any protein bands (data not shown). A cluster of bands, denoted Srp (Figure 4, lane 1, arrow and arrowhead), had a slightly slower mobility compared with *in vitro* translated Srp, which migrates at ~130 kDa (Figure 4, lane 2). Pre-treatment of the extract with potato acid phosphatase shifted the mobility of these bands to the same as that of the *in vitro* translated product, suggesting that Srp is phosphorylated (data not shown). The antisera did not cross-react with any protein in the mock-translated wheat germ extract (Figure 4, lane 3); therefore, we conclude that the 130 kDa protein band corresponds to full-length *in vitro* translated Srp. A band corresponding to a 170 kDa protein, denoted Srp', was also detected by the S2 antiserum in extracts of mbn-2 cells (Figure 4, lane 1). The same protein bands were visualized using the antibody S1, directed against another domain of Srp, as S2 (data not shown). Therefore, these bands cannot be due to cross-reactivity of the antisera with an unrelated protein, but most likely reveal different forms of Srp. The electrophoresis was carried out under denaturing conditions indicating that the 170 kDa protein band is either a covalently modified form of Srp or the translational product of an alternative mRNA. Lossky and Wensink (1995) reported that alternative *srp* mRNA forms exist in different tissues. However, Northern blot analysis with total RNA from mbn-2 cells did not reveal any band that would correspond to the 170 kDa form (data not shown). The 170 kDa protein was not detected in fat body extracts from third instar larvae (data not shown). Therefore, we conclude that this is a haemocyte-specific form of Srp.

Intracellular localization of Srp

The fat body is the main site of antimicrobial peptide synthesis in response to infection (Samakovlis *et al.*, 1990). The expression of *srp* in the embryonic fat body has been reported from stage 5 (Abel *et al.*, 1993; Rehorn *et al.*, 1996; Sam *et al.*, 1996). To investigate the expression of Srp in the fully developed larval fat body, immunostaining of third instar larvae was performed. Strong Srp

Fig. 5. Srp is localized to the nucleus. (A and B) Srp immunostaining of third instar larval fat body (A) and of *mbn-2* cells (B) using the S2 antiserum. (C) Western blot assay. Cytoplasmic (C) (lanes 1 and 2), nuclear (N) (lanes 3 and 4) and total extracts (T) (lane 5) were prepared from untreated (-) (lanes 1, 3 and 5) and LPS-treated (+) *mbn-2* cells (lanes 2 and 4). The lower band denoted Srp corresponds to a 130 kDa protein and co-migrates with *in vitro* translated *srp* (see Figure 3). The upper band denoted Srp' is the 170 kDa form of Srp.

staining was seen in the fat body and the staining was more prominent in the nuclei than in the cytoplasm (Figure 5A). In a parallel independent study, it was demonstrated that Srp is expressed in the larval fat body, gonads, gut, lymph glands and in the pericardial cells (Brodu *et al.*, 1999). Srp was localized in the nucleus in all these tissues.

Immunostaining of *mbn-2* cells demonstrated that Srp is a nuclear protein also in these cells, as was previously found for the GBA. All cells were positive when stained with anti-Srp antiserum, but the intensity of the staining showed some variation (Figure 5B). The Srp staining was more pronounced in the nuclei of some cells (Figure 5B, arrow and arrowhead). To confirm biochemically that Srp is a nuclear protein, Western blot analysis was done on cytoplasmic (Figure 5C, lanes 1 and 2) and nuclear extracts (Figure 5C, lanes 3 and 4) of *mbn-2* cells. Srp was present predominantly in the nuclear fractions. Neither the localization nor the intensity of the Srp-specific bands was affected by addition of LPS before harvesting the cells (Figure 5C). We conclude that Srp is a nuclear protein, present in tissues where antimicrobial peptides are being produced.

The larval fat body contains nuclear GBA

To investigate if the presence of Srp in fat body nuclei correlates with the existence of factors that bind to the GATA sequence, we performed EMSA with nuclear extracts of fat body dissected from third instar larvae. Two different GATA-binding activities (GBA-1 and GBA-2) appeared when fat body extracts were incubated with a 32 P-labelled GATA oligonucleotide (Figure 6A, lane 1). Both GBA-1 and GBA-2 were competed effectively by an unlabelled GATA oligonucleotide (Figure 6A, lane 2), but not by the mutated oligonucleotide, m1 (Figure 6A, lane 3). To identify the presence of Srp in GBA-1 and GBA-2, the fat body nuclear extracts were pre-incubated with antisera S1 and S2. Pre-treatment of the fat body nuclear extracts with the S1 antiserum disrupted both GBA-1 and GBA-2 (Figure 6B, lane 3). Pre-treatment of

Fig. 6. GATA-binding activities in larval fat body extracts. (A) EMSA with nuclear extract from third instar larval fat body (lanes 1–3) and a 32 P-labelled GATA probe (wt). Unlabelled competitors were added to the reactions, wt in lane 2 and m1 in lane 3. The protein–DNA band indicated by an open circle is due to unspecific binding. The complex indicated by an arrowhead is an unidentified GATA-binding activity found in some extract preparations. (B) EMSA with nuclear extract from third instar larval fat body (lanes 1–3) and a 32 P-labelled GATA probe (wt). Nuclear extract was pre-incubated with antisera against the C-terminus of Srp (S2) (lane 2) or against the DNA-binding domain of Srp (S1) (lane 3). The asterisk indicates the supershift of the GBA-1 (lane 2). To minimize unspecific binding, unlabelled m1 oligonucleotide was added to the binding reactions.

the extracts with the S2 antiserum resulted in a supershift of the GBA-1 (Figure 6B, lane 2, asterisk), while the GBA-2 remained unaffected by this antiserum (Figure 6B, lane 2). We suggest that two forms of Srp exist in larval fat body, and that one form thereof (GBA-2) does not contain the C-terminal 22 amino acids, the epitope to which the S2 antiserum is directed. The mobilities of GBA-1 and GBA from *mbn-2* cell nuclear extracts were similar (data not shown). Both complexes reacted with the S1 and S2 antisera, suggesting that GBA-1 and GBA are the same form of Srp.

Srp activates the *CecA1* promoter

It was demonstrated previously that the GATA motif is necessary for full *Drosophila CecA1* promoter activity in transfection assays of *mbn-2* cells (Kadalayil *et al.*, 1997). In order to test if Srp can *trans*-activate the *Drosophila CecA1* promoter, co-transfection assays were performed. The *srp* cDNA was inserted into the expression vector pAct5C and co-transfected with the pA10 or pA16 reporter constructs in the *mbn-2* cell line (Figure 1). Increasing amounts of the expression plasmid pAct-*srp* were transfected with a constant amount of reporter plasmid pA10, and led to significantly increased levels of β -gal activity in the cell extracts (Figure 7A). Co-transfections of 2 μ g of expression plasmid pAct-*Srp* and the reporter plasmid pA16 (Figure 1), carrying a mutated GATA motif, yielded 35% β -gal activity as compared with pA10 (Figure 7A).

Fig. 7. Srp activates *Drosophila CecA1* expression. **(A)** β -Gal expression in co-transfection assays. mbn-2 cells were co-transfected with 1 μ g of reporter plasmid pA10 and increasing amounts of expression plasmid pAct-srp as indicated, or with 1 μ g of reporter plasmid pA16 and 2 μ g of pAct-srp. The cells were incubated with (grey bars) or without (white bars) LPS (10 μ g/ml) 4 h prior to harvest. The results shown are the average of at least four independent experiments with standard deviation indicated as T-bars. **(B)** β -Gal activity in extracts from transgenic larvae carrying the *CecA1-lacZ* reporter construct pA10, and UAS-Srp driven by the heat shock-inducible hs-Gal4⁸⁹⁻²⁻¹, or the enhancer trap GAL4 line c729, which constitutively express GAL4 in the fat body. Heat shock (+HS) was carried out at 37°C for 1 h and larvae were allowed to recover for 3 h.

Addition of LPS to the media before harvesting the cells did not significantly increase the β -gal activity (Figure 7A, grey bars) in comparison with the unchallenged cells (Figure 7A, white bars). The results from the co-transfection experiments were confirmed by utilizing the GAL4/UAS system to overexpress Srp *in vivo* (Figure 7B). The level of β -gal activity in extracts from transgenic larvae carrying pA10, UAS-Srp and hs-GAL4 showed a 2- to 3-fold enhancement when the larvae were subjected to heat shock as compared with untreated animals. Enhanced β -gal activity was also found in larvae carrying pA10, UAS-Srp and the GAL4 enhancer trap line c729, which constitutively expresses GAL4 in the fat body. The activity was 2- to 3-fold higher as compared with larvae carrying pA10 alone (Figure 7B). We conclude that Srp acts as a positive regulator of the *Drosophila CecA1* promoter and therefore is likely to be a key regulator of antimicrobial peptide gene expression.

Discussion

Several conclusions can be drawn based on the results presented in this study. (i) The GATA motif is essential for expression of the *CecA1* gene in larval fat body but dispensable for the infection-dependent expression in adult fat body. (ii) The GATA transcription factor Srp is expressed and localized in the nucleus of immunocompetent tissues. (iii) Srp shares DNA-binding properties with the GBA found in mbn-2 cell and larval fat body nuclear fractions. (iv) Two different antibodies directed towards different parts of Srp reacted with the GBA by destroying the complex or causing it to shift in a DNA-binding assay. (v) Overexpression of Srp in larval fat body and in mbn-2 cells led to activation of the *CecA1* promoter in the absence of infection. These results led us to conclude that Srp is the main component of the GBA.

The *in vivo* importance of the GATA motif

The GATA sequence is present in proximity to a κ B site in all the known regulatory regions of antimicrobial genes in *Drosophila melanogaster* and in several other insects as well. In an earlier study, we have shown by transfection assays that the GATA sequence is needed for proper *CecA1* promoter activity in the mbn-2 cell line (Kadalayil *et al.*, 1997). In this study, we show for the first time that the GATA sequence is essential for expression of an immunity gene *in vivo*. Our results show that the GATA motif is needed for the *CecA1* gene to be expressed properly in the larval fat body during infection (Figure 2B). Our results also clearly demonstrate that the expression of the *CecA1* gene in the adult fat body is not dependent on the GATA motif (Figure 2E and G). A number of other genes show a similar switch in their dependence on a GATA motif regarding their fat body-specific expression. The ecdysone-regulated fat body protein 1 (*Fbp1*) gene, which is expressed in the larval fat body, contains essential GATA motifs within its promoter (Brodu *et al.*, 1999). *Fbp1* is not expressed in the adult stage. Expression of the *Drosophila Adh* gene in the larval fat body is dependent on a GATA motif within the proximal promoter (Abel *et al.*, 1993). In the adult, the fat body expression of the *Adh* gene takes place from the distal promoter where no GATA motif has been found, while the proximal promoter is silenced by the adult enhancer factor (AEF) (Ren and Maniatis, 1998). Also, the expression of the yolk protein genes, *Yp1* and *Yp2*, in the female adult fat body is not dependent on the GATA motifs present in their regulatory regions, although the motifs are essential for the ovary-specific expression (Lossky and Wensink, 1995). Expression of the drosocin gene, coding for another antimicrobial peptide in *Drosophila*, has also been found to be controlled by separate elements in the larva versus the adult (Charlet *et al.*, 1996). In transgenic flies, 2.5 kb of drosocin upstream sequence was sufficient to drive high levels of LPS-inducible reporter gene expression in adult fat body but not in larval fat body. Addition of a 3' genomic region from the drosocin gene resulted in increased transcription levels in larval fat body upon infection, similar to those of the endogenous drosocin gene (Charlet *et al.*, 1996). Interestingly, we have noted that this 3' region contains a GATA motif in close proximity to a κ B site, indicating the possibility that high levels of drosocin expression in

larval fat body are regulated via the nested GATA/κB site present in the downstream region.

The compositions of proteins present in the fat body at different developmental stages presumably is not the same. Larval and adult fat bodies are two different tissues with distinct developmental origins (Hoshizaki *et al.*, 1995). Studies of mutations affecting the *Drosophila* immune response have often been carried out solely in larvae or in adults, with the risk of overlooking a possible disparate effect of the mutation at the two developmental stages.

The GBA in *mbn-2* cells is Srp

We have suggested Srp to be a strong candidate as a regulator of the antimicrobial peptide genes in *D.melanogaster* based on the biochemical properties of the GBA. We show in this study that *in vitro* translated Srp can bind to the *CecA1* GATA motif. The binding properties of Srp are equivalent to the GBA in that both need the GATA (core) sequence for binding and that the GC on the 3' side of the motif is dispensable (Figure 3A; Kadalayil *et al.*, 1997). The Srp-DNA complex and the GBA also have the same apparent mobility when separated by electrophoresis in a native polyacrylamide gel (Figure 3A). This study is the first to explore the DNA-binding properties of the full-length Srp. Other *in vitro* DNA-binding studies have demonstrated that bacterially expressed ABF (a shorter form of Srp) can bind to the GATA site within the *Adh* proximal promoter (Abel *et al.*, 1993) and to the GATA sites regulating the ovary-specific expression of the *Yp1* and *Yp2* genes (Lossky and Wen-sink, 1995).

One of the most compelling pieces of evidence that Srp participates in the regulation of the antimicrobial peptide genes is the demonstration that two different anti-Srp antibodies react with the GBA (Figure 3B). The GATA sequence to which the GBA binds is found in the regulatory regions of all the four *Cec* genes, and in the dipterizin and the metchnikowin genes (Charlet *et al.*, 1996; Kadalayil *et al.*, 1997; Levashina *et al.*, 1998). We propose that Srp is a key regulator of all the *Drosophila* antimicrobial peptide genes in the larval fat body. The GATA motif is also present in upstream regions of antimicrobial peptide genes in other insects (Kadalayil *et al.*, 1997). In *Bombyx mori*, a GATA transcription factor, BmGATAβ, has been reported to be expressed in the fat body (Drevet *et al.*, 1995). BmGATAβ could be a possible regulator of antimicrobial peptide genes in *B.mori*.

Srp expression in immunocompetent tissues and Srp activity

Srp RNA has been reported to be expressed throughout development, with the highest levels in the embryo. *In situ* hybridization experiments in embryos have revealed that *srp* is expressed in the embryonic fat body and haemocytes (Abel *et al.*, 1993; Rehorn *et al.*, 1996; Sam *et al.*, 1996). The embryonic expression of Srp protein has been followed by immunostaining (Riechmann *et al.*, 1998). So far no studies have shown that the embryonic fat body is immunocompetent. Recent data suggest that antimicrobial peptide genes can be induced in the embryonic epidermis from stage 16 (E.Roos, T.Önfelt and Y.Engström, unpublished). Expression of the *CecA1* gene in the fat body was observed from the first larval instar

and throughout development. Our results demonstrate that Srp protein is expressed in third instar larval fat body and in the *mbn-2* cell line (Figure 5). In the *mbn-2* cell line, we found one major form of GBA that specifically binds to the GATA sequence, while two different forms of GBA are present in the larval fat body. Two different anti-Srp antibodies reacted with GBA-1, demonstrating its identity as Srp. GBA-2 was only recognized by the S1 antiserum. We suggest that GBA-2 contains a fat body-specific form of Srp, which lack the C-terminal 22 amino acids.

We found that Srp is a constitutively nuclear protein, unlike the Rel proteins that reside in the cytoplasm and are translocated to the nucleus after infection. Our current model is that Srp is present in the larval fat body and haemocyte nuclei and bound to DNA, keeping it in an open conformation prior to infection. When an infection takes place, the Rel proteins are translocated to the nuclei and bind to the κB motif, leading to instant expression of the antimicrobial genes in the larval fat body. Our results show that Srp can activate the *CecA1* promoter via the GATA motif when co-transfected into *mbn-2* cells, which suggests that Srp is a positive *trans*-activator of the *Drosophila* antimicrobial peptide genes (Figure 7A). Furthermore, overexpression of Srp in larvae led to expression of the *CecA1* reporter construct, showing that Srp acts as an activator of an immunity gene *in vivo*. We did not observe any increased β-gal activity when the transfected cells were treated with LPS, indicating that Srp *trans*-activation does not respond to LPS signalling. We conclude that Srp is needed for tissue-specific *CecA1* promoter activity, and suggest that Srp is a competence factor for genes expressed in the larval fat body and haemocytes.

Further studies will be needed to understand fully the regulation of the antimicrobial peptide genes at different developmental stages. In the adult fat body, other as yet unknown factors may have a function similar to Srp, although it is possible that the Rel proteins are able to turn on the antimicrobial peptide gene expression in adults without help from other specific transcription factors. In addition to the GATA protein Srp, factors binding to other potential regulatory regions, such as the GAAANN, NF-IL6 and the region 1 sequences (Georgel *et al.*, 1993, 1995; Engström, 1998), could conceal other competence factors that together with the Rel proteins regulate expression of immunity genes in different tissues. How the antimicrobial peptide genes are regulated in haemocytes at different developmental stages is not well understood. The role of the GATA motif and of Srp in haemocytes from larvae and adults currently is being investigated. In mammals, several GATA transcription factors have been found and redundancy amongst the factors has been reported (Pevny *et al.*, 1995). A possible involvement of *pnr*, dGATAc and/or other as yet unidentified GATA transcription factors in *Drosophila* immunity cannot yet be excluded. However, *pnr* is not expressed in the larval fat body (J.-A.Lepesant, personal communication) and therefore it is unlikely that *pnr* has a role in this tissue. It is possible that *pnr* regulates antimicrobial gene expression in other tissues, stressing the importance of further studies on the involvement of GATA transcription factors in immune-related functions.

Materials and methods

Antibodies

The rabbit-antiserum denoted S1 was made against amino acids 279–680 of Srp, including the GATA type Zn finger, and purified as described in Sam *et al.* (1996). The rabbit antiserum denoted S2 is directed against the C-terminal 22 amino acids of the Srp protein (Hu, 1995).

Recombinant DNA

The *srp* expression plasmid pAct-*srp* was constructed by inserting a 3.4 kb *Bgl*II-*Eco*RI fragment into the *Drosophila* expression vector pAct5C-PL (Krasnow *et al.*, 1989). The single-stranded DNA ends in the *srp* fragment were trimmed with T4 DNA polymerase to generate blunt ends. The vector was opened in the polylinker with *Eco*RV and treated with calf intestinal phosphatase, before ligation to the *srp* fragment using T4 DNA ligase.

Construction of pA10 and pA16 has been described previously (Engström *et al.*, 1993; Kadalayil *et al.*, 1997). For P-element transformation, the *Xho*I-*Xba*I fragment of the *Cec-lacZ* fusion pA16 was moved from the pBSlac20 vector into the P-element vector pW8 (Klemenz *et al.*, 1987).

P-element-mediated transformation and β -galactosidase staining in tissues

Transformations were done according to Rubin and Spradling (1982).

Transgenic flies and third instar larvae were injected with LPS (10 μ g/ml). After 3–4 h, the adults were rinsed in 95% ethanol and opened up using forceps and needles, and then placed in individual wells of a 96-well microtitre plate. Larvae were opened at the anterior end, turned inside out with the help of forceps and placed in individual wells. The dissected adults and larvae were fixed in 0.5% glutaraldehyde in phosphate-buffered saline (PBS) pH 7.3 for 20 min, rinsed in PBS and stained in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) as described in Engström *et al.* (1992).

Overexpression of Srp in vivo

The GAL4/UAS system (Brand and Perrimon, 1993) was used to study the effects of Srp overexpression. Transgenic flies carrying the *CecA1-lacZ* reporter construct pA10 were crossed with UAS-Srp flies and hs-Gal4⁸⁹⁻²⁻¹ (generated in the laboratory of A.Brand) or the enhancer trap GAL4 line *c729* (generated by K.Kaiser). Heat shock was carried out at 37°C for 1 h and larvae were allowed to recover for 3 h. Total cell extracts were generated from ~20 larvae after first freezing the larvae on dry ice and then homogenizing in 100 μ l of 20 mM HEPES pH 7.9, 0.56 M KCl, 0.2 mM EDTA, 1.5 mM MgCl₂ supplemented with protease inhibitor cocktail according to the manufacturer (Boehringer Mannheim). The homogenates were left on ice for 45 min. Cell debris was removed by centrifugation at 13 000 r.p.m. for 15 min in a microcentrifuge. β -Gal activity was measured at 420 nm using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate (Sambrook *et al.*, 1989).

Immunofluorescence

The immunocytochemistry of mbn-2 cells was investigated as described in Engström and Rozell (1988) and of larval fat body as described in Cantera and Nässel (1992). The Srp antiserum S2 was diluted 1:200 for all immunostainings.

Cell cultures and transfection of cells

Drosophila mbn-2 cells (Gateff *et al.*, 1980) were grown at 25°C in Schneider's medium (LABFAB) supplemented with 5% fetal calf serum (Gibco-BRL), 1.8 g/l stable L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin and 50 μ g/ml gentamycin (Pansystems, GmbH).

Transfection by the calcium phosphate precipitation method (Rio and Rubin, 1985) and measurements of relative β -gal activity were done according to Kadalayil *et al.* (1997).

Cell extracts

Extracts were prepared from 10⁷ mbn-2 cells. An immune response was activated by the addition of purified LPS (10 μ g/ml) from *Escherichia coli* strain 055:B5 to the cells 1 h before harvest. Nuclear and cytoplasmic extracts from untreated or LPS-treated mbn-2 cells were prepared according to Grant *et al.* (1992). To generate total cell extracts, the cells were first washed twice in PBS pH 7.3, pelleted and dissolved in 3 vols of 20 mM HEPES pH 7.9, 0.56 M KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM benzamidine, 50 μ g/ml trypsin inhibitor and 25% glycerol. The

cells were lysed by freezing in liquid N₂ and thawing on ice while pipetting up and down 10 times. The lysates were left at 4°C for 1 h, the pipetting was repeated and finally the lysates were centrifuged to remove cell debris.

Fat body extracts

Canton-S third instar wandering larvae were hand-dissected in PBS, pH 7.3. Fat bodies were frozen immediately on dry ice. Fifty fat bodies were homogenized in 100 μ l of 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol (DTT). The homogenate was kept on ice for 15 min before being lysed by addition of 0.6% (v/v) NP-40. Nuclei were pelleted by centrifugation at 13 000 r.p.m. for 5 min in a microcentrifuge. The nuclear pellet was resuspended in 50 μ l of 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 10% (v/v) glycerol. The nuclei were lysed by shaking at 4°C for 30 min. The lysates were centrifuged at 13 000 r.p.m. for 15 min to remove cell debris. Protease inhibitor cocktail (Boehringer Mannheim) was added to all buffers according to the manufacturer's instructions.

Western blot assay

Cell extracts containing 30 μ g of protein or 5 μ l of *in vitro* translated *srp* were separated in a 7.5% SDS-polyacrylamide gel at a constant current of 20 mA. Proteins were transferred to a Hybond-C membrane (Amersham) by electrophoresis in a mini vertical gel system (E-C Apparatus Co.) at 15 V constant voltage for 90 min at room temperature. The membrane was blocked in 0.2% casein in TBST (10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.5% Tween-20) and then incubated with antiserum S1 (1:10 000) or with antiserum S2 (1:25 000) in TBST. Incubation with alkaline phosphatase-conjugated goat anti-rabbit serum (pre-adsorbed against *Drosophila* mbn-2 cells) (1:10 000) (Sigma), washing and development of the alkaline phosphatase reaction were carried out according to Blake *et al.* (1984).

Electrophoretic mobility shift assay

Deoxynucleotides were labelled with [α -³²P]dCTP and the Klenow DNA polymerase. The oligonucleotides used were: wt, 5'-d(gacaaaatgacAGATAAGGCatgc); m1, 5'-d(aacaaaatgacACGAGAGGCatgc); and m2, 5'-d(aacaaaatgacAGATAAGTGCatgc). Upper case letters refer to the *Drosophila CecA1* GATA motif. Underlined bases in m1 and m2 indicate the altered nucleotides of the GATA site. We refer to the sequence GATAA indicated in bold as the GATA core sequence. The binding reaction was carried out by mixing 1 ng of ³²P-labelled GATA wt probe, 10 μ g of nuclear extract or 2 μ l of the *in vitro* translated product, 1 μ g of poly(dI-dC) and 60 μ g of bovine serum albumin (BSA) in 20 μ l of EMSA buffer (100 mM NaCl, 15 mM HEPES, 0.75 mM EDTA, 1 mM DTT and 8% glycerol). In competition experiments, 250 ng of unlabelled wt, m1 or m2 oligonucleotide was mixed with the binding reaction. After incubation at room temperature for 15 min, the electrophoresis was carried out on a 5% native polyacrylamide gel. The gel was pre-run at 11 V/cm for 60 min at room temperature in 1× TBE (90 mM Tris-borate and 2 mM EDTA pH 8.0). Electrophoresis was carried out under the same conditions for 90 min. The gel was dried and exposed to a phosphor screen and scanned with a PhosphorImager (Molecular Dynamics).

Extracts treated with antiserum or normal serum were pre-incubated with 1 μ l of the respective antiserum for 10 min at room temperature prior to addition of the probe.

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